

Influence of Mild Hypothermia on Hypoxic-Ischemic Brain Damage in the Immature Rat

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ABSTRACT. Recent studies in adult animals have shown that even small decreases in brain or core temperature ameliorate the damage resulting from hypoxic-ischemic insults. To determine the effect of minor reductions in ambient temperature either during or after an hypoxic-ischemic insult on the brain of the immature rat, 7-d-postnatal rat pups underwent unilateral common carotid artery ligation followed by exposure to hypoxia in 8% oxygen for 3 h. Control animals were maintained at 37°C during hypoxia-ischemia. Intrischemic hypothermia was induced during the insult at temperatures of 34°C and 31°C. Postischemic hypothermia was induced by exposing rat pups that underwent hypoxia at 37°C to recovering environments of 34°C and 31°C. Temperatures were recorded every 15 min from thermistor probes placed in the ipsilateral hemisphere and rectally. Neuropathologic alterations were assessed at 30 postnatal d. During hypoxia, animals became poikilothermic. Brain damage occurred in 90% of rat pups exposed to hypoxia-ischemia at 37°C. Cerebral injury significantly decreased with decreasing temperatures during hypoxia-ischemia ($p < 0.01$). Only 30% of rats had brain damage when exposed to hypoxia-ischemia at 34°C, and none of the rats exposed at 31°C had brain damage. In contrast, there was no difference in the extent of cerebral injury between rat pups recovered under hypothermic conditions of either 34°C or 31°C compared with those recovered at 37°C. The results indicate that reductions in temperature of 3 to 6°C have a protective effect during but not immediately after hypoxia-ischemia. These findings have important implications about the thermoregulatory control of the sick newborn infant. (*Pediatr Res* 34: 525-529, 1993)

It is well recognized that profound hypothermia (temperature $<30^{\circ}\text{C}$) markedly reduces the brain damage associated with cardiac arrest, and for that reason is frequently used during the operative repair of congenital cardiac lesions (1, 2). More recently, several studies in adult animals have suggested that even mild reductions (2 to 6°C) of either brain or core temperature during reversible forebrain ischemia also significantly decrease the extent of neurologic injury (3-7). Moreover, postischemia there appears to be a "therapeutic window" of up to 30 min duration, during which time the introduction of mild hypother-

mia also is protective (8, 9). Conversely, hyperthermia either during or after an hypoxic-ischemic insult worsens ultimate brain damage (10, 11).

In contrast to the adult, the human term infant, under physiologic circumstances can maintain thermal neutrality only over a severely restricted range of environmental temperatures (12-15). Infants, who are frequently exposed to a variety of "stresses" (birth asphyxia, hypoxia), are usually rendered incapable of even this restricted temperature control (heterothermia). Therefore, environments, which are by definition neutral, can be detrimental under some circumstances. Given the important role thermoregulation plays in the modern-day care of the sick newborn, the present set of experiments was designed to determine the effect of mild systemic hypothermia applied either during or after hypoxia-ischemia on perinatal brain damage.

MATERIALS AND METHODS

Pregnant Wistar rats, purchased from a local breeder, were individually housed and fed *ad libitum*. Rat pups, delivered vaginally, were suckled with their dams until the day of experimentation. The effect of mild hypothermia on brain injury was assessed either during or after cerebral hypoxia-ischemia at environmental temperatures of 37°C (control) and 34°C or 31°C (hypothermia).

PRELIMINARY STUDIES

Preliminary studies were performed to determine the relationship between brain and core (rectal) temperature when environmental temperatures were altered either during hypoxia-ischemia or recovery. Seven-d postnatal rat pups underwent permanent unilateral right common carotid artery ligation under halothane anesthesia (4% induction; 1% maintenance). Neither common carotid artery ligation alone nor hypoxia alone causes a reduction in cerebral blood flow (16) or evidence of cellular injury to the ipsilateral hemisphere (17). Thermistor probes (model 511, Yellow Springs Instruments, Yellow Springs, OH) measuring 0.024 inches in diameter were inserted into the brain to a depth of 2 mm through a burr hole positioned 1.5 mm to the right of the sagittal suture and 2 mm anterior to the lambdoid suture. The probe was then sutured firmly to the scalp, and care was taken to ensure that the skull was completely covered by resuturing the scalp flap in place. A second probe was then placed rectally to a depth of 0.5 cm and taped to the tail. The animals were gently swaddled to minimize movement and possible dislocation of the probes and were allowed to recover from anesthesia for 2 to 3 h under a warming lamp at nesting temperature. The total duration of surgery lasted no longer than 15 min.

After recovery from surgery, the correlation between brain and core temperature during hypothermic hypoxia-ischemia was determined. Individual rat pups were placed in 500-mL glass jars

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immersed in water baths held constant at either 31°C, 34°C, or 37°C ± 0.5°C. The animals were allowed to equilibrate with the environment for 30 min, after which hypoxia was induced by venting 8% O₂-balance nitrogen through inlet/outlet portals. After 3 h of hypoxia-ischemia, the rat pups were recovered in the water baths for an additional 3 h at the preset temperature of either 37°C, 34°C, or 31°C.

The relation between brain and core temperature during post-ischemic hypothermia was assessed by exposing recovered carotid-artery ligated rat pups to hypoxia (8% O₂-balance nitrogen) for 3 h at a constant temperature of 37 ± 0.5°C, after which the animals were placed in water baths set at either 37°C, 34°C, or 31°C for an additional 3 h.

During all preliminary studies, brain and core temperatures were recorded from each animal: 1) after stabilization during normoxia, 2) every 15 min during hypoxia-ischemia and the immediate recovery period (30 min), and 3) every 30 min thereafter for up to 3 h.

After the experimental procedure, all rat pups were killed and cerebral thermistor probe placement confirmed by direct visualization of the probe after coronally sectioning the fresh brain. Data from only those animals that survived and in which the probe placement was within the caudate-putamen were considered valid. The information obtained from these preliminary studies then was used in the design of the definitive experimental protocol.

EXPERIMENTAL PROTOCOL

Intra- and posthypoxic-ischemic hypothermia. To determine the consequence of mild hypothermia on neuropathologic outcome during or after hypoxia-ischemia, separate groups of 7-d-postnatal rat pups underwent right common carotid artery ligation with rectal probe placement as described above (probes were not inserted into the brain). The animals then, were allowed to recover under a warming lamp set at nesting temperature (34.5 ± 0.5°C) for 2 to 3 h, after which they were placed in the 500-mL jars and exposed to hypoxia (8% oxygen - balance nitrogen) at either 37°C, 34°C, or 31°C for 3 h. The rectal probes were then removed, and the pups were returned to their dams and allowed to grow to 30 d postnatal age, at which time they were killed for neuropathologic assessment.

In experiments to determine the effect of postischemic hypothermia, 7-d-postnatal rat pups were prepared surgically as described above and exposed to hypoxia at 37°C for 3 h. Immediately after the insult, the pups were recovered in water baths preset at temperatures of 37°C, 34°C, or 31°C in room air for 3 h of recovery. All animals were then returned to their dams to 30 d of age for subsequent neuropathologic assessment.

Neuropathologic assessment. Brain damage was examined at 30 d postnatal age (23 d of recovery). The present model provides for a spectrum of cerebral injury ranging from normal to severe brain infarction ipsilateral to the common carotid artery ligation (17). To accurately assess and compare the extent of brain damage, a system of neuropathologic rank scoring was used (18).

Each rat pup was given a lethal injection of pentobarbital (100 mg/kg) intraperitoneally, after which its brain was rapidly removed from its skull and immersed in solutions of formaldehyde, acetic acid, and methanol (1:1:8). Individual rat brains then were examined by two independent observers blinded to the treatment category. Visual inspection of the brains displayed a range of damage from 1) normal-appearing brains in which no discernable difference in the size of the two cerebral hemispheres was noted; 2) mild damage in which the lateral-posterior aspect of the right hemisphere was atrophic; 3) moderate damage indicating brains in which both the anterior and posterior aspects of the right hemisphere were atrophic; and 4) severe damage referring to the total destruction, by prior infarction, of the cerebral cortex in the distribution of the middle cerebral artery of the ipsilateral hemisphere. The observers then graded the extent of damage in

each animal by rank scoring each brain from least to most damaged. Each brain was compared with the entire group and ranked accordingly.

All brains in the intras ischemic groups were further assessed histopathologically. Cerebra were sectioned at the level of the anterior commissure and posteriorly through the mammillary bodies. Sections were stained with hematoxylin and eosin, and examined histologically. Brains with mild damage were noted to have evidence of selective neuronal necrosis. Infarcted brains displayed pan-necrosis of all tissue elements with cystic infarction. Histologic sections from each animal were compared and again rank ordered from least to most damaged.

Statistical analysis. Comparisons of core and brain temperature were made by linear regression analysis. Rank scoring of data for assessment of neuropathologic outcome were obtained nonparametrically using the Mann-Whitney *U* test. Analysis of variance with Dunnett's correction was applied where appropriate. Significance was considered at $p < 0.05$.

RESULTS

Preliminary studies. Seven-d postnatal rats displayed partial homeothermic control under normoxic environmental conditions. At an environmental temperature of 37 ± 0.5°C, the animals maintained their core temperatures at 36.6 ± 0.6°C, whereas at a temperature of 34°C or 31°C, core temperatures were maintained at 34.8°C or 34.6°C, respectively ($p < 0.05$ compared with normothermia).

With the onset of hypoxia-ischemia, the temperature of all rat pups rapidly changed to reflect that of their environmental temperature (Fig. 1). During recovery from hypoxia-ischemia, two patterns of thermal regulation were evident. Rat pups exposed to hypoxia-ischemia at 34°C or 31°C (and therefore either not damaged, or only mildly damaged) (see below) reacquired their ability to thermoregulate almost immediately on recovery from hypoxia-ischemia (Fig. 1), their core/brain temperatures returning to values more closely reflecting that of their preischemic interval. In contrast, those animals exposed to hypoxia-ischemia at 37°C, (rat pups that uniformly displayed severe damage) and who were placed in cooler temperatures postischemically did not return to their preischemic homeothermic state, but rather their core temperatures continued to closely reflect that of the environment indicating a poikilothermic state (Fig. 2).

Brain and core temperatures correlated well during both normoxia and hypoxia-ischemia (Figs. 1 and 2) [$r = 0.75, 0.62$, and 0.66 for 37°C, 34°C, and 31°C groups, respectively, ($p < 0.05$)]. During recovery, the correlation between brain and core temperatures persisted only in those pups exposed to hypoxia-ischemia at 37°C and were recovered at either 31°C ($r = 0.85$; $p < 0.01$).

Intraobserver agreement for visual inspection of the gross neuropathologic injury was very high ($r > 0.95$). Further, there was excellent agreement about the extent of brain damage between cerebral cortices examined visually and histologically ($r > 0.95$).

Mortality and neuropathologic outcome. There were no deaths in either group exposed to hypoxia-ischemia at 34°C or 31°C compared with a 10% mortality during hypoxia-ischemia at 37°C.

Brain damage occurred in 90% of those rat pups exposed to hypoxia-ischemia at 37°C, 70% of which displayed cerebral infarcts by histologic examination (Fig. 3).

To determine whether small variations in temperature were associated with the variability in brain damage seen in this model of perinatal hypoxia-ischemia, mean individual core temperatures of the animals exposed to hypoxia-ischemia at 37°C were compared with their neuropathologic mean rank score. Minor fluctuations around 37°C (±0.5°C) in brain or rectal temperatures did not correlate with the extent of brain damage in this group of animals. Cerebral injury decreased significantly between each

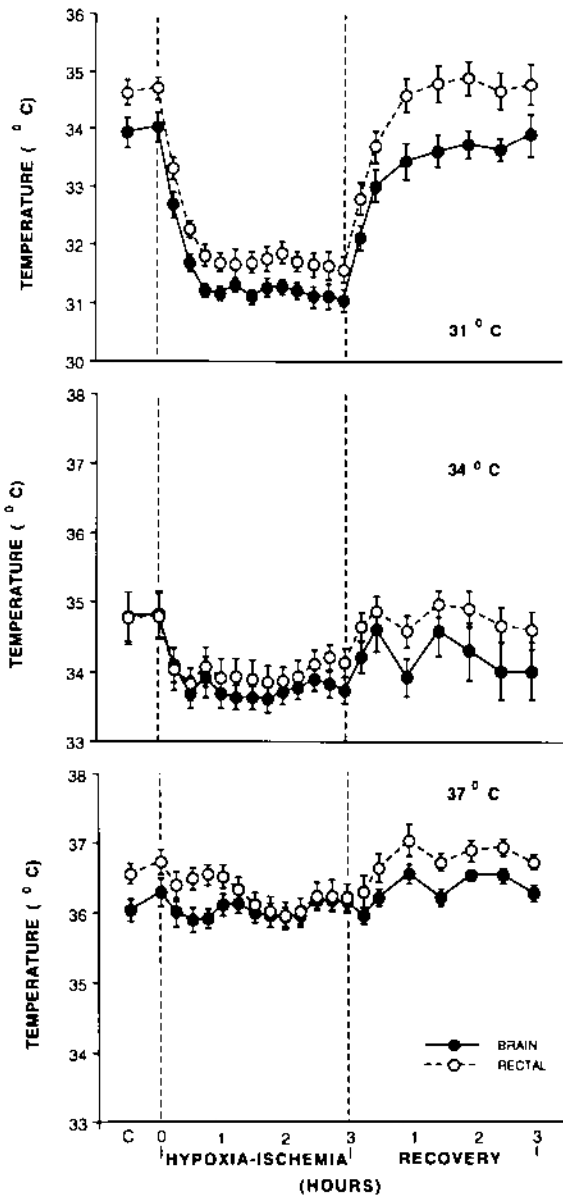


Fig. 1. Brain and core temperature during normoxia, hypoxia-ischemia, and recovery all at either 37°C, 34°C, or 31°C. Values are the means \pm SEM of six to eight animals in each group.

group ($p < 0.01$). At intraischemic temperatures of 34°C, 30% (6 of 21) of the animals displayed neuronal injury, and only one exhibited mild infarction. None of the animals exposed to hypoxia-ischemia at 31°C displayed brain damage.

There was no difference in the extent of brain injury among the groups of rat pups exposed to hypoxia-ischemia at 37°C and recovered at either 37°C, 34°C, or 31°C ($p > 0.75$) (Fig. 4). Therefore, hypothermia induced immediately after hypoxia-ischemia at 37°C provided no cerebral protection.

DISCUSSION

The findings of the present study provide insight into the extent to which 1) the thermoregulatory capability of the immature rat is compromised by hypoxic-ischemic brain injury, and 2) mild hypothermia protects the immature brain during the evolution of hypoxic-ischemic damage.

Our results extend those of Young *et al.* (19), who demonstrated that reducing ambient temperature to 29°C and 21°C partially and completely protects the immature rat brain from cerebral hypoxic-ischemic damage, respectively. Under the pres-

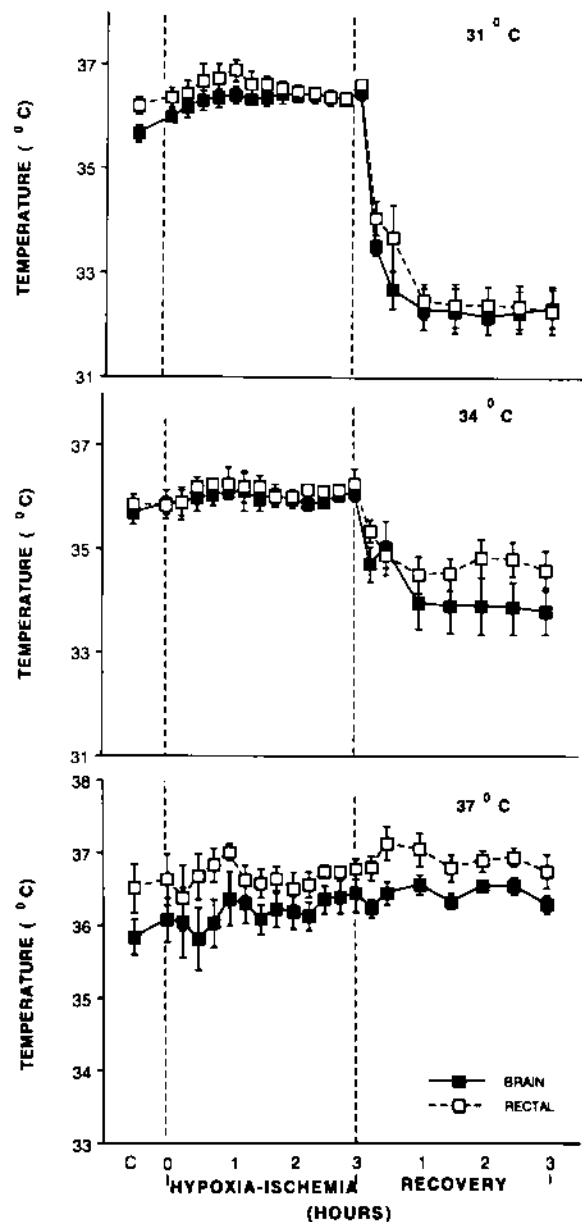


Fig. 2. Brain and core temperature during normoxia and hypoxia-ischemia at 37°C and during recovery at either 37°C, 34°C, or 31°C. Values are the means \pm SEM of six to eight animals in each group.

ent conditions, reductions in body temperature during hypoxia-ischemia by as little as 3°C or 6°C produced near-complete and complete brain protection. Similar results have been obtained in adult animals whereby either mild systemic or selective brain cooling during global or focal cerebral ischemia is protective (3, 5, 6, 19).

In adult animal models of cerebral ischemia, postischemic induction of moderate hypothermia also has been shown to ameliorate brain damage. A reduction in brain temperature from 36°C to 30°C within 30 min after bihemispheric cerebral ischemia significantly reduced the pyramidal neuron damage of the CA₁ sector of the hippocampus. Beneficial effects are no longer present when moderate hypothermia is delayed beyond 30 min of recovery (8, 20). Similar cerebral protective effects have been shown after total cerebral ischemia produced by cardiac arrest and even after experimental traumatic brain injury (21, 22). In contrast, postischemic hypothermia induced immediately after the insult in our immature rat model of cerebral hypoxia-ischemia provided no benefit to the developing brain.

The mechanism by which intraischemic hypothermia exerts

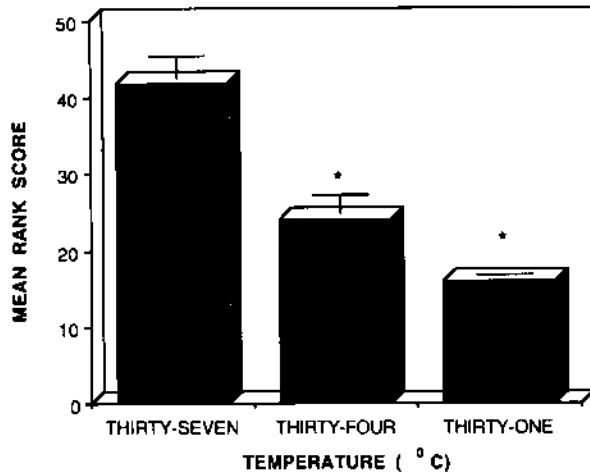


Fig. 3. Mean rank score of brains of rat pups exposed to intras ischemic hypothermic hypoxia-ischemia at 37°C ($n = 16$), 34°C ($n = 21$), and 31°C ($n = 16$). The mean score of brains assessed as being normal was 16, with values >16 having evidence of cerebral damage. Values represent \pm SEM. *, $p < 0.01$ compared with controls at 37°C.

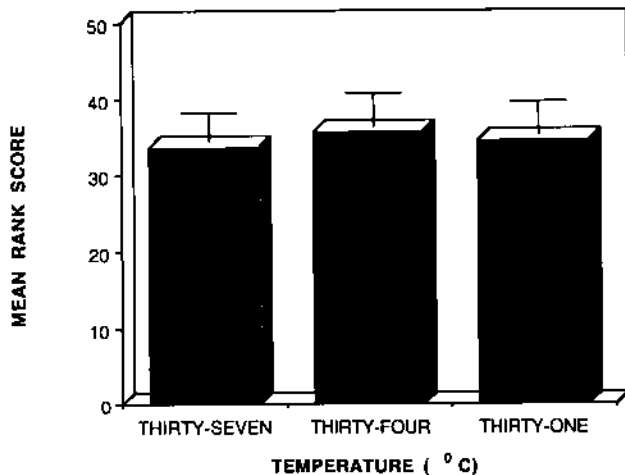


Fig. 4. Mean rank score of brains of rat pups exposed to hypoxia-ischemia at 37°C, followed by the induction of either normothermia (37°C, $n = 22$) or mild hypothermia (34°C, $n = 20$ or 31°C, $n = 26$) during recovery for 3 h. Values represent means \pm SEM.

its protective effect remains unknown. Several investigators have implicated a role for hypothermia in attenuating excitatory amino acid (glutamate, aspartate) neurotoxicity during hypoxia-ischemia (23, 24) and in the prolongation of the extracellular accumulation of the inhibitory neurotransmitter glycine (23, 25). Whether a similar mechanism for the protective effect of hypothermia is at play in the immature brain remains in question. Few studies have documented an increase in glutamate afflux from the axon terminal into the extracellular space during hypoxia-ischemia in the developing brain. Gordon *et al.* (26) found only a variable 3-fold elevation of extracellular glutamate and aspartate during hypoxia-ischemia in the 7-d-old rat compared with the greater than 60-fold increase generally seen in adult brain undergoing ischemic stress (27). Under simulated ischemic conditions, Cherici *et al.* (28) were unable to document an increase in glutamate or aspartate release from prepared immature rat hippocampal slices compared with a dramatically enhanced release of these neurotransmitters from similarly prepared adult hippocampal slices.

The complete absence of a protective effect of mild hypothermia after hypoxia-ischemia was an unexpected finding. In our study, core/brain temperatures began to fall within minutes of

the onset of the recovery period. By 15 min postischemia, brain temperature had reduced by 1.3°C and 3.0°C in the 34°C and 31°C hypothermic groups, respectively. Though it is possible that a more rapid reduction in brain temperature may have provided some protection, the absolute lack of any difference in outcome among the three groups clearly makes this unlikely.

In adult animals, monitoring of core and brain temperature has shown that a transient hyperthermia (approximately 1.0°C) occurs postischemia (9, 29). Thus, it has been postulated that maintaining body and brain normothermic or inducing mild hypothermia during early recovery can partially protect the previously ischemic brain from damage by inhibiting the post-ischemic rise in temperature (8, 9). In the present study, an elevation above normal of either core or brain temperature did not occur during recovery. Whichever accompanying pathophysiologic alterations occur during the period of postischemic hyperthermia in the adult, therefore, clearly do not play a role in the immature rat during recovery.

The effect of mild hypothermia in reducing the cerebral metabolic rate during hypoxia-ischemia and recovery and its role in cerebral protection remain controversial. Contrasting data from experiments performed in adult animals show a sparing of high energy phosphate depletion during hypothermic ischemia on the one hand (7, 30, 31) but an inability to preserve optimal cellular ATP on the other (3, 32). The effect of mild hypothermia on energy metabolism of the immature brain during hypoxia-ischemia remains unknown.

On recirculation after hypoxia-ischemia, only a partial recovery of cerebral high-energy phosphate reserves occurs in the 7-d-old rat (33), whereas in the adult brain rendered ischemic there is an initial complete restoration of energy reserves followed by a secondary decline (34–36). This early replenishment of ATP presumably is required for the reestablishment of membrane potentials necessary for the survival of those neurons and glia adversely affected during the ischemic phase but that have the potential to recover (37). Given that a reduction in temperature of even a few degrees inhibits cerebral metabolic rate (38), the lack of protection afforded by posthypoxic-ischemic hypothermia in our study might be due to an inability of ATP to recover. Without an immediate restitution of energy stores and a chance for restabilization of cell membranes, those cells potentially recoverable will die.

The findings of the present animal investigation may have important implications about the human situation. In the newborn human infant, earlier studies pertaining to the effect of environmental temperature on outcome suggested that infants maintained in a warm environment displayed a reduced mortality compared with those maintained at cooler temperatures (39–42). As a result, current practice emphasizes the maintenance of thermoneutrality in the management of the sick human newborn infant. Whereas prolonged hypothermia has obvious detrimental effects (22, 43–45), maintenance of neutral temperatures, irrespective of the condition of the newborn infant, may be harmful. Given the present experimental findings, circumstances appear to exist in which the judicious intermittent use of mild hypothermia might improve neurologic morbidity of the compromised newborn infant. Additional studies are required to more specifically delineate this potential therapeutic intervention.

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